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Nitrate Respiration in Chemoautotrophic Symbionts of the Bivalve *Lucinoma aequizonata* Is Not Regulated by Oxygen

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The marine bivalve *Lucinoma aequizonata* has intracellular chemoautotrophic symbionts residing in the gill tissue. These bacteria are capable of nitrate respiration even under fully saturated oxygen conditions. Nitrate reductase in the symbionts of *L. aequizonata* appears to be constitutively expressed and without significant regulation by oxygen or nitrate. We discuss the stationary-phase growth state of the symbionts as an explanation for the lack of enzyme induction.

Denitrification and nitrate respiration have been reported for many hundreds of phylogenetically diverse bacteria (19). These bacteria use nitrate as a terminal electron acceptor alternative to oxygen in bacterial respiration. Nitrate respiration is generally an anaerobic process. Oxygen regulates the electron flow in the respiratory chain by diversion of electrons (12); it controls nitrate uptake (10, 11) and subunit assembly (6) and regulates the synthesis of anaerobic respiratory enzymes on the level of trancription and translation (14, 15, 24, 25).

An increasing number of bacteria are known to denitrify in the presence of oxygen (17, 23). While there is unequivocal evidence that aerobic denitrification occurs, very little is known about the underlying mechanisms. With respect to the multitude of inhibitory effects of oxygen, one would expect complex regulatory mechanisms.

Aerobic nitrate respiration has also been documented in chemoautotrophic symbioses, e.g., the symbionts of the hydrothermal vent tube worm *Riftia pachyptila* (9) and the clam *Lucinoma aequizonata* (8). A population of *L. aequizonata* (*Lucinidae*) inhabits the Santa Barbara Basin (3), where the local oxygen concentration in the seawater is extremely low (below 5% saturation) and the extensive mud burrows inhabited by the clams become anaerobic below the first few millimeters. Nitrate is present in the seawater at about 30 μM.

The symbionts of *L. aequizonata* respire nitrate even under fully aerobic conditions (8). The study presented was designed to investigate how nitrate respiration by the symbionts is different from that of most other bacteria and to identify the mechanisms by which aerobic nitrate respiration is made possible. For that purpose, we have compared nitrate respiration rates and enzyme activities under aerobic and anaerobic conditions in intact animals and purified symbiont suspensions.

L. aequizonata and mud from the sampling site were collected at a depth of 510 ± 10 m by Otter trawl in the Santa Barbara Basin, California. Upon return to the laboratory, mud basins were set up in flowthrough aquaria (8°C), in which the animals were maintained. All experiments were performed within 2 months following collection.

Nitrate respiration. Purified symbionts (4) (50 μl) were incubated in YSI (Yellow Springs Instruments) chamber glass vials which were closed with a rubber septum in 10 ml of 0.2-μm-filtered seawater (with or without added nitrate). Incubations were made anaerobic by bubbling nitrogen gas through an injection needle prior to and during the experiment. Aerobic conditions were established by bubbling continuously with air.

Nitrate respiration rates were determined by measuring the concentration of nitrite in the supernatant of aliquots of the suspension which had been drawn through an injection needle at 15-min intervals and centrifuged (1 min at 14,000 × *g*). Anaerobic rates were about two to three times higher than aerobic rates (see Fig. 2B).

When the aerobic incubations of symbionts were made anaerobic and vice versa, the nitrate respiration rates adjusted to the new incubation conditions after a lag of about 20 min (Fig. 1). Symbiont respiration clearly does not come to an immediate halt, as has been documented for *Escherichia coli* (12) and *Pseudomonas* spp. (2, 11). If electron transport to oxygen is not present in the symbiont respiratory chain, then the electron flow cannot be redirected to cytochrome oxidase. A time lag would be expected.

The oxygen respiration of bacterial suspensions was measured with a sulfide-insensitive Clarke type oxygen electrode (Strathkelvin Instruments, Glasgow, United Kingdom) in a respiration chamber (Strathkelvin Instruments) at room temperature with continuous stirring in 1.95 ml of 0.2-μm-filtered seawater containing 50 μl of freshly purified symbiont suspension. No oxygen consumption was measurable in symbiont preparations which were able to respire nitrate. Oxygen respiration was not detectable after addition of different substrates (sulfide, thiosulfate, and organic acids) or different buffers (10 mM MOPS [morpholinopropanesulfonic acid] and 10 mM imidazole) or at different incubation temperatures (8, 15, and 21°C).

In order to characterize the symbiont respiratory chain, typical enzymes of bacterial respiration were measured. Enzyme activities were determined in the quick-frozen symbiont pellet which had been resuspended in a Dounce homogenizer in enzyme buffer (100 mM phosphate, 5 mM nitrate [pH 7.4]) and sonified twice for 15 s each time. Nitrate reductase was determined by the method of Nicholas et al. (18), and cytochrome *c* oxidase was measured by the method of Hand and Somero (7). Different reductases were tested photometrically by the assay described by Kalman and Gunsalus (13). The protein concentration was determined with the bicinchoninic acid assay and reagents (Pierce, Rockford, Ill.).

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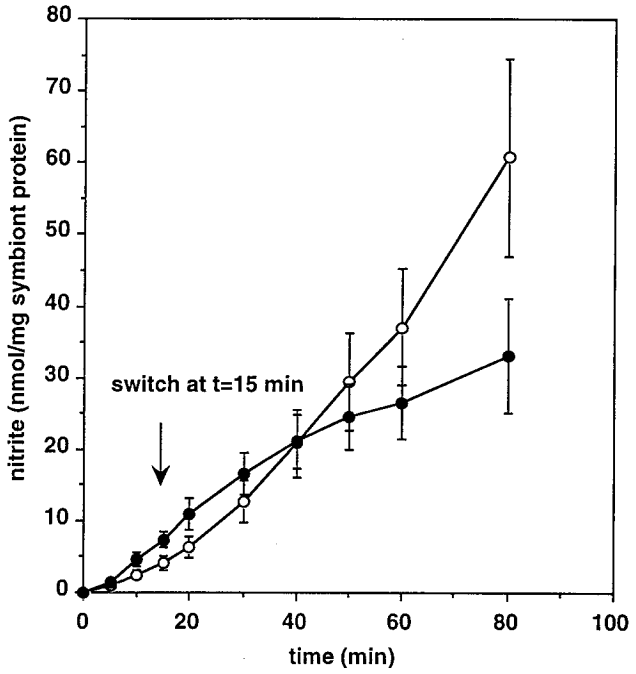

FIG. 1. Nitrate respiration in incubations of purified symbionts of *L. aequizonata* in seawater containing 1 mM nitrate. ●, initially anaerobic incubation; ○, initially aerobic incubation. The arrow indicates when the incubation conditions were switched from anaerobic to aerobic and vice versa. Data are given as mean ± standard error (n = 3).

Cytochrome oxidase was not detectable in four purified symbiont suspensions. However, it was present in the foot tissue of *L. aequizonata* (0.3 ± 0.3 μmol per g [wet weight] per min, n = 3). Of the four reducing enzymes measured in purified symbiont preparations, TMAO reductase activity was highest (800 to 1,000 nmol/mg of protein/min, n = 5), followed by nitrate reductase (80 to 300 nmol/mg of protein/min, n = 3). Fumarate and dimethyl sulfoxide reductase (n = 5) were not detectable. None of the enzymes were present in the mantle, used as a control tissue (n = 2). TMAO reductase is involved in anaerobic respiration by facultatively anaerobic bacteria. The metabolic role of the remarkably high TMAO reductase activities in the symbionts is unclear at this time.

Induction of symbiont nitrate reductase. Nitrate respiration can be regulated at the level of enzyme synthesis. Oxygen is known to inhibit the expression of nitrate reductase at the level of transcription and translation (5, 15, 27). Whether or not the synthesis of this enzyme is inhibited by similar mechanisms in aerobic denitrifiers is not known. In order to compare the symbiotic bacteria with known nitrate reducers, we measured, as a first approach, enzyme activities in purified symbionts and whole animals to determine if and how nitrate reductase is inducible.

There was no increase in nitrate reductase activities during inducing (anaerobic plus nitrate) incubations (Fig. 2A). The symbionts were capable of considerable aerobic nitrate respiration (Fig. 2B). Because there was considerable variation between the different preparations (12 to 51 nmol of nitrite per mg of protein per min), the data are expressed as a percentage of the initial activity. These results suggest that the enzyme is expressed constitutively under the experimental conditions used. When the symbionts were incubated aerobically, the activities decreased to about half of the initial activity. At least two explanations are possible for the decrease. First, the enzyme itself may be inactivated through contact with oxygen. However, other nitrate reductases are reportedly quite stable in the presence of oxygen (6, 20, 24), and *E. coli* nitrate reductase was stable in our experiments (Fig. 2C). Alternatively, intracellular degradation may exceed the synthesis of nitrate reductase under these conditions.

One possible explanation for the lack of nitrate reductase induction could be the slow protein turnover due to the symbionts' nongrowing state, since the overall rates of protein synthesis during the stationary phase, with the exception of specific stress proteins and others, are generally greatly reduced (16). This hypothesis is based on comparative experiments with *E. coli* K-12 strain W3110 (27) grown in Luria-Bertani broth (1% tryptone, 1% NaCl, 0.5% yeast extract). This strain was chosen as a control because its nitrate reductase regulation has been well characterized. However, only a few studies have correlated the induction of nitrate reductase with growth rate, and the results are contradictory (1, 14, 20, 21).

When we incubated stationary-phase *E. coli* (1 mg of protein per ml) anaerobically in the presence of nitrate, there was no increase in nitrate reductase activities (Fig. 2C). In contrast, logarithmically growing cells displayed induction within one doubling time, as described by others (24, 25) (data not shown). If the growth rate itself is responsible for a different phenotypic expression of the enzyme, then it would point to a more complicated connection than was previously assumed.

Our results obtained with purified symbionts were confirmed in experiments with whole clams (Table 1). Experiments with whole animals were done in batches of 30 clams, which were maintained in 4 liters of seawater at 6°C under control (aerobic sea water) and inducing conditions, which were established by bubbling sea water with nitrogen in the presence of 1 mM nitrate. At various time points, six animals were killed, and the concentration of nitrite in the mantle fluid was determined. The gill tissue was rinsed, homogenized in enzyme buffer (100 mM phosphate, 5 mM nitrate [pH 7.4]), and filtered through 600-μm Nitex filters.

Enzyme activities in whole clams did not change under inducing conditions (anaerobic plus nitrate) or in aerated sea water for 9 days (n = 3) (Table 1). The addition of an electron donor (50 μM sulfide or 100 μM thiosulfate), the choice of shorter time intervals, and starting the incubations immediately on board ship did not change the results. To account for changing concentrations of symbionts in the gills, bacterial numbers in formalin-fixed aliquots were determined by epifluorescence after staining with 4,6-diamidino-2-phenylindole (DAPI) (1 μg/ml of extract) (22). The data were then calculated as nanomoles of nitrite produced per number of bacteria per minute. However, the results remained unchanged (data not shown).

Nitrite accumulated in the mantle water of animals maintained under inducing conditions (Table 1) but only in traces when the animals were kept in seawater or anaerobically without nitrate. At these concentrations, nitrite was not inhibitory to enzyme induction (data not shown).

The results presented by us can be explained in view of the natural environment of the symbionts. The intracellular conditions inside the bacteriocytes of the animal's gills are likely to be an environment in which the symbionts are exposed to little fluctuation. The lack of oxygen respiration suggests that they may not be exposed to oxygen in vivo. Thus, the symbionts are not required to change their mode of respiration. In addition, the symbionts are in the stationary phase inside the gills, which in itself may be sufficient to explain the lack of nitrate reductase regulation. Thus, we come to the conclusion that symbiont

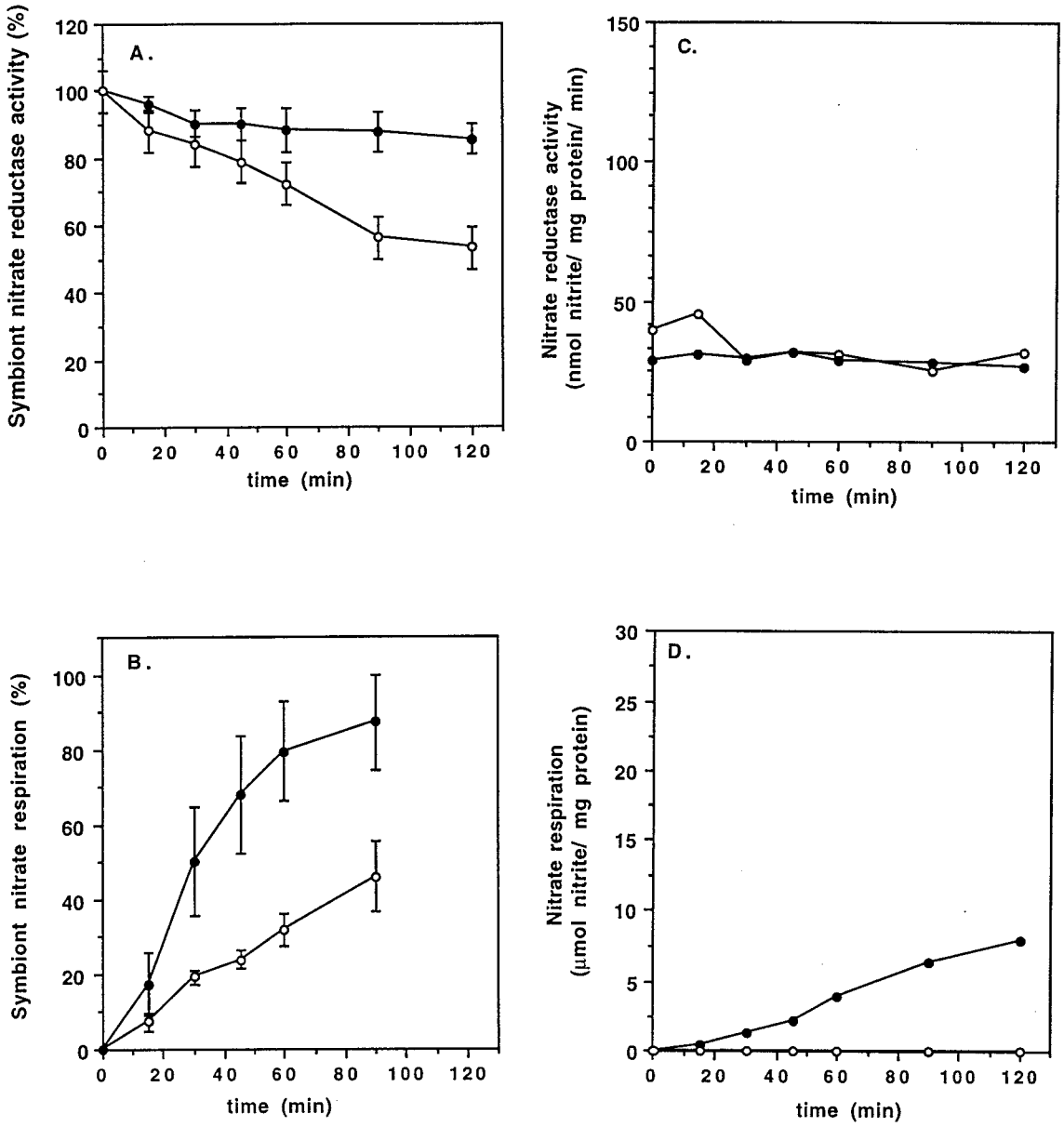

FIG. 2. Nitrate reductase activities and nitrate respiration rates of purified symbionts of *L. aequizonata* (A and B) and *E. coli* in stationary phase (C and D). ○, aerobic; ●, anaerobic plus 10 mM nitrate. Aerobic nitrate respiration rates were measured after addition of 10 mM nitrate to aerobic incubation medium (B and D). To account for variability between different symbiont preparations, the results in panels A and B are presented as a percentage of the initial activity and as the highest respiration rate measured, respectively. (A and B) Data are means ± standard error (n = 4). (C and D) One representative set of three sets of data is shown.

TABLE 1. Nitrate reductase activities and concentration of nitrite in the mantle water of *L. aequizonata* clams maintained in aerated seawater and anaerobically with 1 mM nitrate for 9 days

Day	Aerobic incubation (n)		Anaerobic incubation with 1 mM nitrate (n)	
	Enzyme activity (nmol mg^{-1} min^{-1})	Nitrite in mantle water (μM)	Enzyme activity (nmol mg^{-1} min^{-1})	Nitrite in mantle water (μM)
0	3.5 ± 0.8 (8)	0 (8)	3.5 ± 0.8 (8)	0 (8)
2	0.7 ± 0.2 (6)	2.5 ± 0.9 (6)	2.8 ± 1.6 (6)	265 ± 88 (6)
5	7.7 ± 2.1 (6)	6.3 ± 0.8 (6)	3.6 ± 1.7 (5)	451 ± 189 (5)
9	3.0 ± 1.1 (6)	8.9 ± 1.7 (6)	2.0 ± 0.6 (6)	499 ± 270 (6)

nitrate reductase is constitutively expressed in vivo and without significant regulation at the level of enzyme synthesis. *L. aequizonata* is thus uniquely adapted to its habitat through its symbiosis with bacteria which respire nitrate but not oxygen.

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